Development of an effective antibacterial treatment mehtod in orthodontic patients. Clinical and experimental studies

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Zentrum für Zahn-, Mund- und Kieferheilkunde der Universität Zürich

Klinik für Kieferorthopädie und Kinderzahnmedizin

Direktor: Prof. Dr. med. dent. Timo Peltomäki

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Clinical and experimental studies

Kumulative Habilitationsschrift
zur Erlangung der Venia legendi der medizinischen Fakultät der Universität Zürich

vorgelegt von

Rengin Attin, Dr. med. dent., geborene Tütüncü

Zürich, Dezember 2008

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Zugrunde liegende Originalarbeiten:

1. Attin R, Tuna A, Attin T, Brunner E, Noack MJ. Efficacy of differently concentrated chlorhexidine varnishes in decreasing Mutans Streptococci and Lactobacilli counts. *Arch Oral Biol.* 2003; 48:503-509

2. Attin R, Thon C, Schlagenhauf U, Werner C, Wiegand A, Hannig C, Attin T. Recolonization of mutans steptococci on teeth with orthodontic appliances after antimicrobial therapy. *Eur J Orthod.* 2005; 27:489-493

3. Attin R, Ilse A, Werner C, Wiegand A, Attin T. Antimicrobial effectiveness of a highly concentrated chlorhexidine varnish treatment in teenagers with fixed orthodontic appliances. *Angle Orthod.* 2006; 76:1022-1027

4. Attin T, Abouassi T, Becker K, Wiegand A, Roos M, Attin R. A new method for chlorhexidine (CHX) determination: CHX release after application of differently concentrated CHX-containing preparations on artificial fissures. *Clin Oral Invest.* 2008; 12:189-196

Introduction

Caries preventive measures, based on good oral hygiene, establishment of non cariogenic dietary habits and the regular supplement of fluorides often are not sufficient to prevent the formation of new carious lesions in patients with high caries activity. Beyond a certain caries activity level neither an increase in the frequency of tooth brushing nor an increase in the dosage of administered fluoride are suitable to effectively stop the demineralization process in high-risk individuals.[1] Patients undergoing orthodontic therapy have oral ecological changes which lead to increased numbers of *mutans streptococci* (*ms*) in saliva and plaque.[2,3] It has also been shown that orthodontic treatment with fixed appliances may result in enamel demineralization and an increased number of carious lesions.[4] Therefore, preventive efforts in these risk groups concentrate on the direct suppression of the cariogenic micro-flora by chemotherapeutics. Chlorhexidine is the most potent documented antimicrobial agent against *mutans streptococci* and dental caries. Different modes of administration are recommended for caries prevention.[5-7] It has been suggested that, compared with other forms of application[7,8], chlorhexidine varnish application results in a longer-lasting suppression of *mutans streptococci*. There are different varnish application systems with different concentrations available on the market. Therefore, the aim of the present series of studies was to evaluate the best strategy of *mutans streptococci* suppression with chlorhexidine varnishes in patients undergoing orthodontic therapy with fixed appliances.

Materials and Methods

In the first experiment, a randomized controlled clinical study including 24 patients with high levels of *mutans streptococci* in saliva was performed to evaluate the most effective chlorhexidine varnishes on the market which were discussed as potent antimicrobials against *mutans streptococci* (study no. 1). In the following studies the effects of the most potent varnish on orthodontic patients were examined. First of all we wanted to know the reasons for a possibly reduced effect of this varnish in patients with fixed appliances. In a clinical randomized controlled study with spilt mouth design we compared the recolonization pattern of *ms* on densely colonized teeth with and without appliances after treatment with a highly concentrated chlorhexidine varnish (study no. 2). In the third clinical study the recolonization pattern of *ms* was examined in patients (n=19) with high bacterial counts in saliva and fixed orthodontic appliances after a treatment with highly concentrated chlorhexidine varnish (study no. 3). In study no. 4, a new in vitro method for determination of chlorhexidine release after application of differently concentrated chlorhexidine-containing preparations was performed. The release of chlorhexidine after 0-7 days was determined.

Results

The use of the highly concentrated 40% chlorhexidine varnish (EC 40®) in patients with high *ms* levels was significantly more effective than that of a lowly concentrated varnish (study no. 1).

This was, therefore, examined in the following experiments in orthodontic patients. The degree of recolonization with *mutans streptococci* after varnish treatment was significantly higher on teeth with orthodontic brackets (study no. 2). The therapy with EC 40® in orthodontic patients with fixed appliances resulted in a very quick recolonization with *ms*.

After 2 weeks the *ms* values returned to baseline values (study no.3). The in vitro study (study no. 4) revealed a continuous chlorhexidine release from enamel specimens with the highest increase for EC 40® in comparison to the chlorhexidine varnish Cervitec®.

Discussion

The *ms* counts in the clinical studies were evaluated with a commercially available *ms*- test, namely the chair-side Strip mutans® test. The reliability of this method has been proven by numerous studies.[9,10] There is a significant correlation between the conventional analysis with MSB agar and the Strip mutans® test.[9,10] Intra-individual differences with the Strip mutans® method were investigated by El-Nadeef and Bratthall.[11] They observed that repeated tests performed in one subject usually showed no significant discrepancy. Only in very rare cases tests varied in more than one category. So it is safe to say that the Strip mutans® method is a very reliable method with good handling properties. In study no.1 the authors were able to establish that there are significant differences in the inhibitory effect of *ms* of the tested highly and lowly concentrated chlorhexidine varnishes. The highly concentrated varnish led to a distinct reduction of *ms* over a period of up to 12 weeks. These results encouraged the authors to examine the highly concentrated varnish in high risk patients, which can be said to include patients with fixed appliances. As it had also been shown that the application of fixed appliances in orthodontic treatment favours enamel demineralization and leads to an increased number of carious lesions.[4] It has hitherto been assumed that insertion of orthodontic devices increases caries risk, since fixed orthodontic appliances provide artificial niches for cariogenic microorganisms. This was confirmed in study no.2. Sugar restriction has been shown to diminish the occurrence of S. mutans in the mouth[12], but there is no evidence that sugar consumption has also an influence on recolonization with S. mutans after antibacterial therapy. Since in study no. 2, using a split mouth design, distinct differences were observed between the arches with and without appliances, it may be assumed that the sugar consumption of the respective individual may only exert a negligible impact on recolonization. But the association between sugar consumption and recolonization of S. mutans after antimicrobial therapy should be assessed in further studies.

Since a primary aim of study no.2 was to evaluate the importance of bands and brackets on the recolonization pattern of *ms* after antibacterial therapy, both the test and the control group was existent in one patient's mouth. Each subject was his/her own control. This way the number of participating subjects could be kept relatively low. Inter-group differences in the average of ms values at baseline were negligibly small and not significant. Study no. 2 revealed that ms colonization was higher in teeth receiving fixed orthodontic appliances compared to control teeth without appliances. In this sense it should be noted that studies performed in high risk orthodontic patients did not find significant differences in caries increment after repeated application of highly or lowly concentrated chlorhexidine varnishes.[13-15] In the study of Jenatschke et al.[13] varnish application was performed on the day of bracket placement and was repeated every eight weeks while the fixed appliances were in place. The results of study no 2 show that *ms* counts on teeth with bands and brackets returned to baseline values after 8 weeks. Therefore, bearing our results in mind, it

is intelligible that no effect on caries increment could be achieved, since cariogenic bacteria could not be effectively suppressed. It can be assumed that the recolonization must have taken place during this time interval (i.e. 8 weeks) so that recolonization with *ms* and caries development could not be avoided. The commencement of the recolonization with *ms* after antimicrobial therapy in highly colonized teeth was investigated in study no. 3. The authors were able to demonstrate that *ms* counts returned to baseline values after two weeks. This finding may serve as an explanation for no effect on caries increment having been achieved in former studies[14]. On the other hand, an application mode of applying the varnish even more often, such as once a week, is not practicable. A more effective application mode had to be found to hamper the fast recolonization of *ms* in orthodontic patients. It was hypothesized that this resulted in a slower recolonization with *ms*. As a next step, the exact recolonization time after varnishing had to be determined to set an application interval and to suppress *ms* during the entire therapy with fixed appliances. The interest should be focused on the time at which *mutans streptococci* in patients with densely colonized teeth and with fixed orthodontic appliances return to baseline values after treatment with the 40% chlorhexidine varnish EC 40®. A positive effect could be achieved when varnish application before banding and bracketing would be performed. This was examined in an ongoing study, in which high risk patients were selected before bracketing and were treated with the highly concentrated chlorhexidine varnish immediately before the placement of the fixed appliances.[16] With this strategy the authors could achieve a significantly decreased number of *ms* 4 weeks after varnish treatment - compared to *ms* values before varnish treatment. As orthodontic patients usually return to recall treatment every 4-6 weeks it is desirable that suppression of *ms* due to antimicrobial treatment would last for no less than this period of time. In this ongoing study the authors were able to show that the recolonization time could be prolonged so that a significant reduction was still existent after 4 weeks. Due to these results a prevention strategy could be established, which allowed patients to be screened for high caries risk before orthodontic treatment and which permitted the application of highly concentrated varnish immediately before bracketing. During the entire course of orthodontic therapy varnish treatments must be repeated at 4-week-intervals. The effectiveness of the high concentration varnish is explained by its toxic effect so that almost all *ms* are killed in one single application. This effect is assumed to lead to a delayed recolonization compared to treatments with low concentration chlorhexidine gels or varnishes. Another possible explanation for the effectiveness of EC40® could be that with the highly concentrated varnish a chlorhexidine reservoir might be formed in cracks and enamel porosities and that this reservoir is maintained even if the varnish is removed from the tooth surfaces after a contact time of 8 min. Study no. 4 examined this effect with the result that release of EC40® from enamel specimens was higher than other chlorhexidine formulas. The suppressing effect of the varnish probably could be ameliorated by the cleaning modus of the teeth. A full-mouth-disinfection as described for patients with periodontal diseases before varnish application could be a suitable method for a prolonged suppression of *ms*. In summary it can be concluded that with the series of present studies the effect of an antibacterial treatment in orthodontic patients could be distinctly ameliorated. Further studies must prove the effect on caries increment due to this strategy developed with present investigations.

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Efficacy of differently concentrated chlorhexidine varnishes in decreasing *Mutans streptococci* and *lactobacilli* counts

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Antibacterial varnish; Chlorhexidine; Interdental plaque; Saliva; *Mutans streptococci*; *Lactobacilli*

Summary The objective of the present prospective trial was to compare the efficacy of differently concentrated chlorhexidine varnishes (EC40® = 40% chlorhexidine and Cervitec® = 1% chlorhexidine + 0.1% thymol) on levels of *Mutans streptococci* (*ms*), *lactobacilli* (*lb*) and plaque formation in interproximal plaque and saliva. Twenty-four volunteers with a high level of *ms* in saliva were randomized into two groups and treated with the experimental varnishes. Varnish applications were performed in accordance with literature. Over a period of 2 weeks Cervitec® was applied three times and EC40® once or twice, depending on *ms* counts after first application. Four and 12 weeks after final varnish application *ms* in plaque and saliva were evaluated. Furthermore, *lactobacilli* (*lb*) counts in saliva and the effect on plaque formation were recorded. Both varnishes revealed a reduction of *ms* in interproximal plaque and saliva after 4 and 12 weeks. The highly concentrated varnish revealed a significantly stronger reduction of *ms* in plaque and saliva compared with the lowly-concentrated varnish. No effect could be demonstrated on *lb* counts and plaque formation. The results indicate that the chlorhexidine varnishes tested may reduce *ms* in both interproximal plaque and saliva. However, the application of the highly concentrated varnish EC40® results in a higher decrease of *ms* in plaque sites and saliva.

Introduction

Chlorhexidine is the most potent documented antimicrobial agent against *Mutans streptococci* (*ms*) and dental caries. Different modes of administration are recommended for caries prevention.[1–3] It has been suggested, that chlorhexidine application in varnish form results in a longer-lasting suppression of *ms* compared with other forms of application.[1,4] High and low concentrations of chlorhexidine have been reported to reduce the number of *ms* in plaque and saliva[5–7] for considerable periods of time. This long-lasting effect is probably due to the prolonged contact time between varnish and teeth. Highly concentrated varnishes exhibit a pronounced

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chlorhexidine sustained release. Moreover, the concentration can be 10—40 times higher than in other regimens.[8]

Mutans streptococci in proximal sites are highly resistant to chemico-mechanical measures.[9,10] Therefore, it is an important preventive measure to suppress *ms* in interdental sites. Petersson et al.[11] and Twetman and Petersson[12] reported a significant suppression of *ms* in plaque samples collected from interproximal sites over a 3-month-period following two successive applications of Cervitec® (Vivadent, Schaan, Liechtenstein, lowly concentrated varnish containing 1% chlorhexidine diacetate and 1% thymol as active ingredients). Other authors[7,8,13] examined highly concentrated chlorhexidine varnishes as supersaturated solutions of chlorhexidine-di-acetate in ethanol, stabilized by the natural resin sandarac. The optimal chlorhexidine varnish concentration suggested for suppression of *ms* was 40% of chlorhexidine (EC40®, Explore, Nijmegen, Netherlands). They reported that *ms* were significantly suppressed for at least 4 weeks after a single chlorhexidine varnish application. In some treated subjects in their study the bacteria were effectively suppressed even over 6 weeks. This investigation demonstrated that a chlorhexidine varnish was effective in achieving long-term suppression of interproximal plaque, although the varnish was in contact with the tooth surface for only 15 min. Different varnish regimens of chlorhexidine application and concentration are recommended for an effective suppression of *ms*. No direct comparison has been performed between the efficacy of highly and lowly concentrated varnishes exept Heintze and Twetman[14] with the comparison of different chlorhexidine regimens in relation to different restaurative materials. The aim of present investigation was to compare the efficacy of highly and lowly concentrated varnishes on the suppression of *ms* levels in saliva and interproximal plaque, the inhibiting effect on plaque growth and *lactobacilli*.

Materials and methods

Participants

The participants gave informed consent. Fifty-seven volunteers, patients from the university of cologne, were screened and 24 of them (19—31 years) were selected fulfilling the inclusion criteria: high levels of *ms* in saliva, i.e. at least score 2 identified with the chair-side Strip mutans method according to Jensen and Bratthall.[15] Scores 2 and 3 correspond to approximately 10^5 CFU/ml saliva or more. Each participant had a full dentition and there was a mean dental caries experience of 23 decayed and filled surfaces. None of the subjects had detectable frank caries lesions or defective restaurations. Moreover, radiographs did not reveal any lesions at interproximal tooth surfaces.

Study design

Professional tooth cleaning was performed and oral hygiene instructions were given to the participants prior to the study. For baseline examination and further plaque sampling sessions the subjects refrained from all oral hygiene measures for 24 h. At baseline the plaque index,[16] *ms* levels in plaque and saliva and saliva *lactobacilli* counts were recorded. The *lactobacilli* counts in saliva were recorded with the dip-slide method of Larmas[17] (Dentocult *Lactobacilli* Test, Orion Diagnostica, Liechtenstein).

The plaque *ms* scores were determined with the site-specific modified Strip mutans technique as originally described by Wallman and Krasse[18] and modified by Twetman and Frostner.[19] The number of colony-forming units (CFU) with characteristic morphology was screened and scored 0—3. The evaluation was blinded. Hereby, score 0 corresponds to no CFU (*ms* below detection level) score 1 = 1—10 CFU, corresponding to approximately $<10^4$—10^5 CFU, score 2 = 10—100 CFU, corresponding to approximately to 10^5—10^6 CFU, score 3 > 100 CFU, corresponding to $>10^6$ CFU.

Selected teeth for interproximal plaque sampling were isolated with cotton rolls and dried. A small sterile brush was carefully inserted in the interproximal site (mesial and distal of all permanent first molars = 8 sites per subject). Sampled plaque was immediately spread on the roughened side of the plastic strip from a commercially available test kit (Strip mutans, Orion Diagnostica, Finland). The strips were allowed to dry for 5 min in room temperature and were then incubated for 48 h in a liquid medium. The composition of the medium is similar to the composition of mitis salivarius agar, with a sucrose concentration increased to 30%. Addition of a bacitracin disc® from the kit results in a final concentration of 0.36 U of Bacitracin per ml of the medium.[15] Additionally, a saliva Strip mutans test was performed for each participant. After 48 h cultivation in the liquid medium the scores of *ms* in plaque and saliva[15,18] were recorded with the aid of a stereomicroscope with 10—25 magnification.[19]

The 24 subjects were randomly divided into two experimental groups. Twelve subjects were treated with Cervitec® (Vivadent, Schaan, Liechtenstein),

the remaining volunteers were treated with EC40® (Explore, Nijmegen, Netherlands).

Prior to each varnish application teeth were professionally cleaned with rubber cup and pumice paste. The interdental areas were cleaned with unwaxed dental floss. Each quadrant was isolated with cotton rolls and dried with compressed air. EC40® was applied to all teeth with a brush, delivered into the interproximal areas with unwaxed dental floss. The varnish was left in place for 8 min and then removed with a brush and dental floss. Cervitec® was applied under the same conditions on all teeth but was not removed after application. Furthermore, the participants of the Cervitec® group were instructed not to eat for 3 h and not to brush their teeth for 24 h. Cervitec® was applied as described above on three occasions over a 3-week-period.

In the EC40® group the varnish was applied only once and the *ms* values in saliva were assessed after 1 week as described above. In subjects displaying *ms* in saliva with score 1 or more, the varnish was applied a second time. This was the case in 20% of the subjects in the EC40® group.

Four and 12 weeks after final varnish treatment in each group *ms* levels in plaque and saliva, saliva *lactobacilli* and plaque scores were recorded as described above.

Statistical methods

Plaque indices were compared using Wilcoxon test

All microbiological measurements are observed on a grading scale, i.e. the observations are so-called ordered categorical data and thus, standard statistical methods like the *t*-test or the analysis of variance cannot be applied. For the analysis of such data, ranking methods have been developed.[20] Herefore, the original observations are replaced with their ranks. Since only the four gradings 0, 1, 2, and 3 are the possible values of the data, many observations will have the same values which are called ties in statistical literature. When ranking tied observations are given midranks are assigned. Since ranking methods are used for the analysis of the data, it is reasonable to use the means of the ranks to summarize the outcome of the trial in tables and graphs.

The statistical design underlying the observations in our trial is a repeated measures design, i.e. the same patients are repeatedly observed at several time points. The main question in such a trial is whether the time profiles of the rank means for the two treatment groups are significantly different (significance level was set at 5%). If the hypothesis of parallel time profiles is rejected subsequent post hoc analyses are performed by using an appropriate adjustment of the significance level. This means that statistical significant differences are assumed when the time profiles are not parallel. For a detailed description of these methods we refer to Brunner et al.[21]

Results

No significant differences were found in plaque indices. *P*-values were demonstrated in Table 1.

One hundred and seventy-seven interdental sites (92%) of 192 examined at baseline exhibited high levels (scores 2 and 3) of *ms*. At baseline (time point 1) there was no statistical difference between the two treatment groups (Fig. 1).

Statistical analysis was done as described above using rank mean values. For better illustration of the original data the percentage distributions of the bacterial determinations are given in Tables 2–4.

In Fig. 1, the results of site specific plaque samples 4 (time point 2) and 12 (time point 3) weeks after EC40® and Cervitec® application are demonstrated.

A significantly different time profile could be observed ($P = 0.015$) comparing both varnishes. Both treatment regimens resulted in a significant reduction of *ms* when comparing baseline values to time point 2 (4 weeks) after varnish treatment (EC40 $P < 0.0001$, Cervitec® $P = 0.0008$).

In the Cervitec® group, at time point 3 the values returned gradually to baseline values and were not significantly different from time point 1 ($P = 0.165$). In the EC40® group this effect was maintained even 12 weeks after varnish application (time point 1 compared with time point 2, $P < 0.0001$).

In saliva (Fig. 2) both varnishes resulted in a significant decrease of *ms* after 4 and 12 weeks. This means, that the above described suppression of

Table 1 *P*-values of the statistical comparison of EC40 vs. Cervitec and after treatment with either EC40® or Cervitec® with regard to the changes of plaque values from baseline to 4 weeks, baseline to 12 weeks, and 4–12 weeks.

	Baseline—4 weeks	Baseline—12 weeks	4–12 weeks
EC40® and Cervitec®	0.137	0.173	0.036
EC40®	0.083	0.205	0.304
Cervitec®	0.083	0.449	0.061

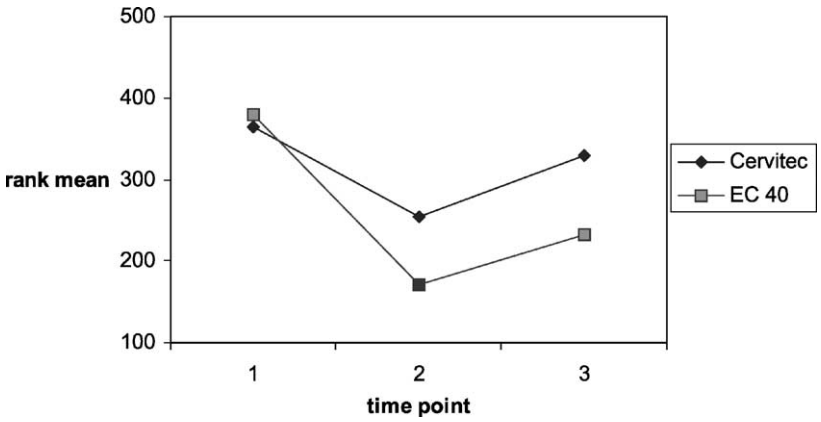

Figure 1 Rank means of mutans counts in plaque after treatment with EC40® and Cervitec® (significantly different time profiles, $P = 0.015$) at various time points (time point 1 = baseline, time point 2 = 4 weeks, time point 3 = 12 weeks).

Table 2 Number of plaque samples with scores (0—3) for *Mutans streptococci* at baseline, 4 and 12 weeks after application of EC40® and Cervitec®.

Mutans streptococci (*ms*) score	EC40®			Cervitec®		
	Baseline	4 weeks	12 weeks	Baseline	4 weeks	12 weeks
0	0 (0)	31 (32)	15 (16)	0 (0)	4 (4)	1 (1)
1	9 (9)	29 (30)	30 (31)	6 (6)	24 (25)	7 (7)
2	24 (25)	20 (21)	24 (25)	36 (37)	47 (49)	48 (50)
3	63 (66)	16 (17)	27 (28)	54 (56)	21 (22)	40 (42)
Total	96 (100)	96 (100)	96 (100)	96 (100)	96 (100)	96 (100)

Values in parentheses are in percent.

Table 3 Number of saliva samples with scores (0—3) for *Mutans streptococci* at baseline, 4 and 12 weeks after application of EC40® and Cervitec®.

Mutans streptococci (*ms*) score	EC40®			Cervitec®		
	Baseline	4 weeks	12 weeks	Baseline	4 weeks	12 weeks
0	0 (0)	4 (33)	4 (33)	0 (0)	0 (0)	1 (8)
1	0 (0)	2 (17)	1 (8)	0 (0)	2 (17)	1 (8)
2	3 (25)	6 (50)	6 (50)	2 (17)	5 (42)	4 (33)
3	9 (75)	0 (0)	1 (8)	10 (83)	5 (42)	6 (50)
Total	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)

Values in parentheses are in percent.

Table 4 *Lactobacilli* counts (in scores) at baseline, 4 and 12 weeks after application of EC40® and Cervitec®.

Lactobacilli (*lb*) score	EC40®			Cervitec®		
	Baseline	4 weeks	12 weeks	Baseline	4 weeks	12 weeks
0	3 (25)	4 (33)	4 (33)	3 (25)	4 (33)	4 (33)
1	2 (17)	2 (17)	1 (8)	3 (25)	2 (17)	2 (17)
2	3 (25)	2 (17)	3 (25)	1 (8)	1 (18)	1 (8)
3	2 (17)	3 (25)	3 (25)	3 (25)	3 (25)	4 (33)
4	2 (17)	1 (8)	1 (8)	2 (17)	2 (17)	1 (8)
Total	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)

Values in parentheses are in percent.

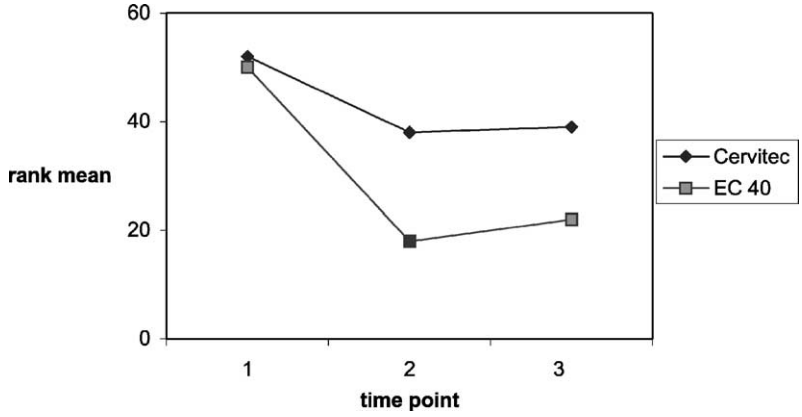

Figure 2 Rank means of mutans counts in saliva after treatment with EC40® and Cervitec® (significantly different time profiles, $P = 0.046$) at various time points (time point 1 = baseline, time point 2 = 4 weeks, time point 3 = 12 weeks).

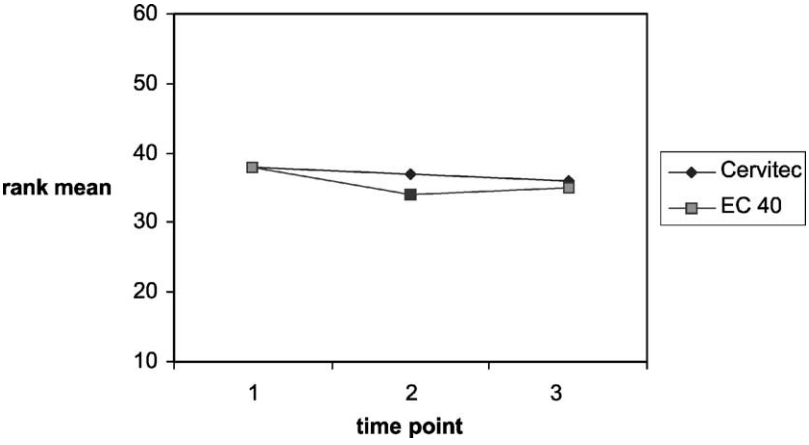

Figure 3 Rank means of *lactobacilli* in saliva after treatment with EC40® and Cervitec® (not significantly different time profiles, $P = 0.772$) at various time points (time point 1 = baseline, time point 2 = 4 weeks, time point 3 = 12 weeks).

ms in plaque at time point 2 was also observed in saliva. The time profiles were significantly different ($P = 0.046$) and EC40® achieved a significantly higher decrease of *ms* in comparison to Cervitec® both in time points 1 and 2.

In both groups (Fig. 3) there was no effect on *lactobacilli* counts in saliva.

Discussion

The results indicate that there are significant differences in the inhibitory effect on *ms* of the tested lowly and highly concentrated chlorhexidine varnishes. The highly concentrated varnish leads to a distinct reduction of *ms* up to 12 weeks. This effect could not be achieved with the lowly concentrated varnish in present study.

It has been shown that *ms* can be suppressed effectively for a prolonged period of time after treatment with highly concentrated varnishes.[8,22,23]

Ie and Schaeken[23] observed that in some patients, two successive applications of highly concentrated varnish is necessary to induce a long time suppression of *ms*. In the present study the first application did not suppress saliva *ms* below detection level in 20% of the patients. In those subjects the highly concentrated varnish was applied a second time. In cases with saliva samples below detection level 1 week after the first application of EC40® the varnish was assumed to have a long-term effect.

The lowly concentrated varnish was generally applied three times. Yet no comparable effect was observed: the present study proved a significant reduction of *ms* in both varnish groups after 4 and 12 weeks. In the high concentration varnish group a significantly higher decrease of *ms* in plaque samples could be observed. This may be due to insufficient chlorhexidine concentration or the insufficient adhesion of the low concentration varnish to the tooth surfaces.

The effectiveness of the high concentration varnish may be explained by a ''radical effect'' of the varnish so that almost all *ms* are killed in a single application. This results in a slower recolonization with *ms* compared to treatments with lowly concentrated agents, such as chlorhexidine gels or varnishes.

With low concentrations of chlorhexidine *ms* may not be killed effectively and proliferate and return to their original numbers within a few weeks.

With lowly concentrated gels the suppression was found to be effective if applied frequently.[3,24] After a single application of 5% chlorhexidine gel *ms* was strongly suppressed for 1 week, but returned to baseline levels within 3 weeks.

Previous studies showed similar findings with lowly concentrated varnishes. When Petersson et al.[11] examined interproximal plaque after treatment with a lowly concentrated varnish (Cervitec®) they found a gradual return to pretreatment values at their 1- and 3-month examination.

Controversal findings are demonstrated in the investigation of Twetman et al.[25] This study resulted in plaque samples with *ms* below detection level even 1 and 6 months after treatment with Cervitec®. This controversial finding can be explained by the different plaque sampling locations. In the present study interproximal plaque was examined, whereas Twetman et al.[25] evaluated *ms* counts in plaque from bracket bases of buccal sites. A better effect in interproximal sites could be expected when Cervitec® is combined with fluoride varnishes.[12,26] In the Cervitec group the dental flossing was forbidden for 1 week after application. With this advice we tried to achieve a longer adhesion of the varnish on teeth and a prolonged recolonization time of *ms*. Of course the adverse effect (increased recolonization in the absence of flossing) could have occurred when flossing had a stronger effect on the colonization of *Mutans streptococci*. There is no indication in literature that flossing without additive substances has an effect on colonization of *ms*. Another possible explanation for the effectiveness of EC40® could be that with the highly concentrated varnish a chlorhexidine reservoir might be formed in cracks and enamel porosities which is maintained even if the varnish is removed from the tooth surfaces after a contact time of 8 min. Since the varnish was removed with a tooth brush and dental floss, it is likely that parts of varnish were left on the surfaces leading to a sustained release with an antibacterial effect on *ms*. In a pilot scanning electron microscope investigation, enamel specimens treated with the high concentration varnish EC40® revealed remnants of the varnish even after mechanical cleaning procedures (not published).

For the application of the lowly concentrated varnish the teeth were isolated with cotton rolls and kept dry while the varnish was applied for further 15 s. The patients were instructed to refrain from eating and drinking for 3 h and from tooth-cleaning for 24 h. Furthermore the use of dental floss was forbidden for the following 7 days. Although these instructions for preserving the varnish on tooth surfaces were rendered, the influence of the tongue and the saliva amount and composition in the lower jaw could be an explanation for the reduced effect of Cervitec® on *ms*.

As published in former studies, early plaque formation could be inhibited by chlorhexidine varnishes.[27] We could not demonstrate an inhibitory effect on plaque formation.

No side effects were noted for the lowly concentrated varnish. In contrast the treatment with the highly concentrated varnish displayed side effects like desquamation of mucosa and taste disturbances in five subjects. These effects disappeared 2—3 days after application.

In previous studies an inhibitory effect of chlorhexidine on *lactobacilli* could not be proven.[20,22,28,29] This was also true for the present study.

It can be summarized and concluded that chlorhexidine varnishes of low and high concentration lead to a reduction of high *ms* values in interdental plaque over a period of 12 weeks. However, the highly concentrated varnish tested, suppressed *ms* in plaque samples more effectively.

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Recolonization of *mutans steptococci* on teeth with orthodontic appliances after antimicrobial therapy

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SUMMARY The aim of the present study was to compare the recolonization pattern of *mutans streptococci* on densely colonized teeth with and without fixed orthodontic appliances after treatment with a 40 per cent chlorhexidine (CHX) varnish (EC 40®, Explore). Healthy subjects free of carious lesions requiring fixed orthodontic appliance treatment but with high bacterial *mutans streptococci* saliva counts were recruited (n = 10). For baseline registration, plaque from buccal sites was sampled and cultivated on Dentocult® strips. Following professional tooth cleaning, CHX varnish was applied to all teeth for 8 minutes. Subsequently, orthodontic brackets and bands were inserted in either the upper or lower arch. Eight weeks after varnish application the degree of recolonization with *mutans streptococci* was reassessed on the buccal sites. Statistical analysis showed that recolonization with *mutans streptococci* was significantly higher ($P < 0.05$) on teeth with orthodontic appliances.

The results indicate that the use of fixed orthodontic appliances creates artificial environments suitable for the proliferation of *mutans streptococci* after CHX varnish suppression.

Introduction

Caries preventive measures, based on good oral hygiene, establishment of non-cariogenic dietary habits and the regular supplement of fluorides, are often not sufficient to prevent the development of new carious lesions in orthodontic patients with high caries activity. Beyond a certain caries activity level, neither an increase in the frequency of tooth brushing nor in the dose of administered fluoride is suitable to effectively stop the demineralization process in high-risk individuals (Øgaard *et al.*, 1994). Patients undergoing orthodontic therapy have oral ecological changes that lead to increased numbers of *mutans streptococci* in saliva and plaque (Lundström and Krasse, 1987a, b).

It has also been shown that the application of fixed appliances results in enamel dermineralization and an increase in the number of carious lesions (Mitchell, 1992). It could be assumed that the insertion of orthodontic devices increases caries risk as they provide artificial niches for cariogenic micro-organisms. Therefore, preventive efforts in these 'at-risk' groups should concentrate on the direct suppression of the cariogenic micro-flora by chemotherapeutics.

Chlorhexidine (CHX) is the most potent documented antimicrobial agent against *mutans streptococci* and dental caries. Different modes of administration have been recommended for caries prevention (Zickert *et al.*, 1982; Fardal and Turnbull, 1986; Emilson, 1994).

It has been suggested that CHX application as a varnish results in a longer lasting suppression of *mutans streptococci* compared with other forms of application (Emilson, 1994; Pienihäkkinen *et al.*, 1995; Attin *et al.*, 2003). High and low concentrations of CHX have been reported to reduce the number of *mutans streptococci* in plaque and saliva (Sandham *et al.*, 1988, 1991; Schaeken *et al.*, 1989) for considerable periods of time (Schaeken *et al.*, 1989; 1991; Schaeken and De Haan, 1989). Numerous studies have examined differently concentrated CHX varnishes formulated as supersaturated solutions of chlorhexidine-di-acetate in ethanol, stabilized by the natural resin sandarac. The optimal CHX varnish concentration suggested for effective suppression of *mutans streptococci* is in 40 per cent CHX (EC40®, Explore, Nijmegen, Netherlands). These studies reported that *mutans streptococci* were significantly suppressed for at least 4 weeks after a single CHX varnish application. This effect was tested in patients not undergoing orthodontic treatment. However, studies performed in high-risk orthodontic patients using highly concentrated CHX varnish treatment resulted in no influence on caries increment (Jenatschke *et al.*, 2001).

It is uncertain whether the recolonization of *mutans streptococci* is enforced by orthodontic devices. The objective of the present study was, therefore, to investigate the recolonization pattern of *mutans streptococci* on sites adjacent to orthodontic appliances in comparison with teeth free of orthodontic devices after antibacterial treatment.

The working hypothesis was that sites adjacent to appliances would have greater colonization than sites without appliances.

Subjects and methods

Subjects

The volunteers gave informed consent for participation in the study. Twenty-seven volunteers requiring fixed orthodontic appliances, at the Department of Orthodontics, were screened and 10 were selected who fulfilled the inclusion criteria: high levels of *mutans streptococci* in saliva, i.e. at least a score of 2 identified with the chair-side Strip mutans method (Jensen and Bratthall, 1989). Scores of 2 and 3 correspond to approximately 10^5 colony-forming units (CFU)/ml saliva or more. While the majority of subjects had a full compliment of teeth, some had undergone premolar extractions due to orthodontic indication and the third molars were unerupted. None of the patients had detectable carious lesions or defective restorations. Moreover, clinical examination and radiographs did not reveal any lesions at the interproximal tooth surfaces.

Study design

Professional tooth cleaning was performed and oral hygiene instruction was given to the participants prior to the study. For the baseline examination, the subjects refrained from all oral hygiene measures for 24 hours. At baseline (time point 1) *mutans streptococci* levels in plaque and saliva were recorded.

The plaque *mutans streptococci* scores were determined with the site-specific modified Strip mutans technique as originally described by Wallman and Krasse (1993) and modified by Twetman (1995). The number of CFU with characteristic morphology was screened and scored 0–3. The evaluation was blind.

Score 0 corresponded to no CFU (*mutans streptococci* below detection level):

score 1 = 1–10 CFU; approximately less than 10^4–10^5 CFU
score 2 = 10–100 CFU; approximately 10^5–10^6 CFU
score 3 > 100 CFU; more than 10^6 CFU

The selected teeth for plaque sampling were isolated with cotton rolls and dried. A small sterile brush was carefully applied to the buccal sites (cervical area) of the two molars to be banded and the premolars to be bracketed and the corresponding teeth in the opposite arch. In subjects with extracted premolars the canines were evaluated. The sampled plaque was immediately spread on the roughened side of the plastic strip from a commercially available test kit (Strip mutans, Orion Diagnostica, Espoo, Finland). The strips were allowed to dry for 5 minutes at room temperature and were then incubated for 48 hours in a liquid medium. The composition of the medium was similar to that of mitis salivarius agar, with the sucrose concentration increased to 30 per cent. The addition of a bacitracin disc® from the kit results in a final concentration of 0.36 U bacitracin/ml medium (Jensen and Bratthall, 1989). Additionally, a saliva Strip mutans test was performed for each participant. After 48 hours' cultivation in the liquid medium the scores of *mutans streptococci* in plaque were recorded with the aid of a stereomicroscope with a magnification of ×10–25 (Twetman, 1995).

Prior to each varnish application with EC40®, the teeth were professionally cleaned with rubber cups and pumice paste and the interdental areas with unwaxed dental floss. Each quadrant was isolated with cotton rolls and dried with compressed air. EC40® was applied to all teeth with a brush, delivered to the interproximal areas with unwaxed dental floss. The varnish was left in place for 8 minutes and then removed with a brush and dental floss. The varnish was applied only once and the *mutans streptococci* values in the saliva were assessed after 1 week, as described above. In subjects displaying *mutans streptococci* in saliva with a score of 1 or higher, the varnish was applied a second time. This was the case in 20 per cent of the subjects.

The orthodontic appliances were randomly placed in either the upper or lower arch of the subject. The respective antagonist teeth in the arch without any appliance served as the control.

Eight weeks after the final varnish treatment, *mutans streptococci* levels in plaque and saliva were recorded at the buccal sites of the control teeth and at the buccal sites adjacent to the brackets, as described above.

Statistical methods

All microbiological measurements were observed on a grading scale, i.e. the observations were so-called ordered categorical data and, thus, standard statistical methods such as the *t*-test or analysis of variance could not be applied. For the analysis of such data, ranking methods have been developed (Brunner and Langer, 2000). For this purpose, the original observations were replaced with their ranks. As only four gradings (0, 1, 2, and 3) were possible, many observations had the same values, which, in the statistical literature, are called 'ties' (Brunner *et al.*, 2002). When tied, the observations were given midranks. Because ranking methods were used for the analysis of the data, it was reasonable to use the means of the ranks to summarize the outcome of the trial in tables and graphs.

The statistical design underlying the observations in the present investigation was a repeated measures design, i.e. the same patients were repeatedly observed at several time points. The main question in such a trial is whether the time profiles of the rank means for the two treatment groups are significantly different (significance level was set at 5 per cent). If the hypothesis of parallel time profiles was rejected, subsequent *post hoc* analyses were performed

using an appropriate adjustment of the significance level. This means that statistically significant differences are assumed when the time profiles are not parallel. For a detailed description of these methods, see Brunner *et al.* (2002).

Results

Statistical analysis was undertaken as described using rank mean values. At baseline (time point 1) there was no statistical difference between teeth prior to active orthodontic treatment and teeth that were not going to have orthodontic intervention, which served as the controls ($P = 0.643$).

The original data for the percentage distributions of the bacterial determinations are given in Figure 1.

The results of site-specific plaque samples on teeth prior to orthodontic treatment and teeth that were not scheduled for orthodontic treatment at baseline (1) and 8 weeks after varnish treatment are shown in Figure 2.

Significantly different time profiles could be observed ($P = 0.023$) when comparing both groups. At baseline, teeth with appliances showed a mean rank of 81, which was not significantly different from the controls (mean rank 72). However, after 8 weeks, the controls exhibited a significantly lower mean rank (32) compared with teeth with orthodontic devices (mean rank 71). For the teeth without orthodontic appliances (controls), varnish treatment resulted in a significant reduction in *mutans streptococci* when comparing the baseline values to those obtained at 8 weeks ($P = 0.0013$). No significant effect could be observed in teeth with orthodontic appliances ($P = 0.218$). For these teeth, the values gradually returned to those found at baseline and were not significantly different from time point 1. Comparing the effect of varnish treatment of teeth with bands versus teeth with brackets after 8 weeks, no significant differences could be observed ($P = 0.183$). Therefore, from Figure 3 it becomes obvious that the mean ranks at baseline (bands: 43; brackets: 47) were not different from the mean ranks 8 weeks after varnish treatment (bands: 38; brackets: 33).

Discussion

It has been shown that following treatment with highly concentrated CHX varnishes, *mutans streptococci* can be suppressed effectively for a prolonged period of time (Schaeken *et al.*, 1991; Ie and Schaeken, 1993; Attin *et al.*, 2003). The optimal concentration has been suggested to be contained in 40 per cent CHX varnishes (Schaeken and De Haan, 1989; Schaeken *et al.*, 1989).

Moreover, Ie and Schaeken (1993) observed that in some subjects two subsequent applications of highly concentrated varnish led to a longer suppression of *mutans streptococci* compared with a single application. This effect was also found in 20 per cent of the subjects in the present study, where the first application did not suppress saliva *mutans streptococci* below the detection level. In these instances, the high concentration varnish was applied for a second time, resulting in effective suppression of *mutans streptococci*. In patients in whom after 1 week the suppression continued to be observed, the varnish was assumed to have a long-term effect.

Because a primary aim of the present study was to evaluate the importance of bands and brackets on the recolonization pattern of *mutans streptococci* after antibacterial therapy, a split mouth design was used, with each subject being his/her own control. In this way, the number of participating subjects could be kept relatively low. Intergroup differences in the average of *mutans streptococci* values at baseline were negligible and not significant.

The present investigation revealed that *mutans streptococci* colonization was higher in teeth with fixed orthodontic appliances compared with the control teeth without appliances. However, this effect was only slightly significant ($P = 0.023$). Therefore, it may not be clinically relevant.

It should be noted that studies performed in high-risk orthodontic patients did not find significant differences in caries increment after repeated application of high or low concentrated CHX varnishes (Lundström and Krasse,

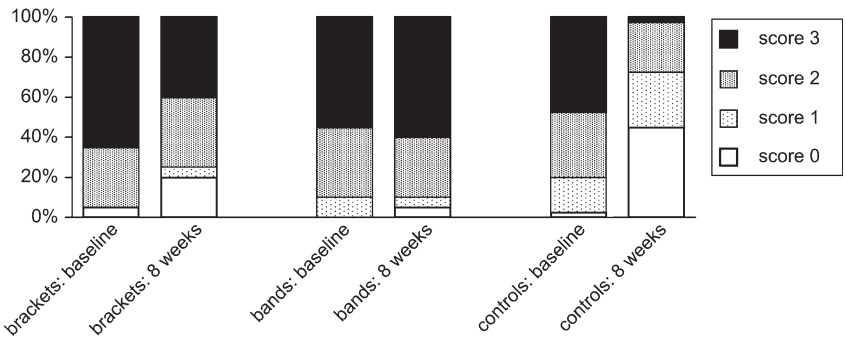

Figure 1 Percentage of plaque samples with scores (0–3) for *mutans streptococci* at baseline and 8 weeks after varnish treatment.

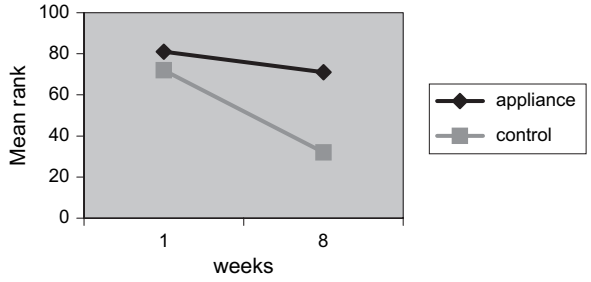

Figure 2 Mean ranks of *mutans streptococci* counts in plaque at baseline and 8 weeks after treatment with EC40® in teeth with or without appliances.

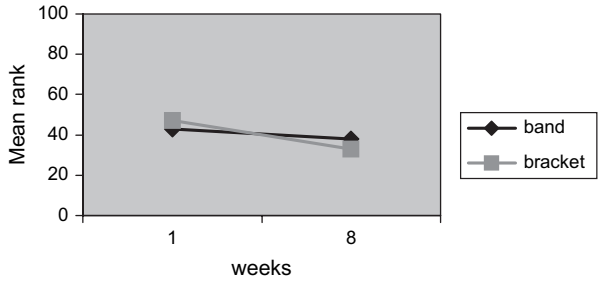

Figure 3 Mean ranks of *mutans streptococci* counts in plaque at baseline and 8 weeks after treatment with EC40® in teeth with bands or brackets.

1987a; Jenatschke *et al.*, 2001). Those authors assumed that the duration of *mutans streptococci* suppression partly depends on the extent to which any retention niches are coated with varnish. It was also speculated that bacterial resistance occurred during continuing varnish therapy.

In the study by Jenatschke *et al.* (2001), varnish application was performed on the day of bracket placement and repeated at 8 week intervals, while the fixed appliances were in place. Furthermore, no second application was performed at the beginning of *mutans streptococci* suppression, as opposed to the present study. The results of the present investigation show that the *mutans streptococci* counts on teeth with brackets and bands returned to baseline values after 8 weeks. Therefore, bearing in mind these findings, it is perceivable that no effect on caries increment could be achieved as cariogenic bacteria could not effectively be suppressed.

The effectiveness of the high concentration varnish may be explained by the toxic effect of the varnish, so that almost all *mutans streptococci* are killed in a single or, as described previously, a two-time application resulting in a delayed recolonization compared with treatment with low concentrated agents, such as CHX gels or varnishes. The presence of orthodontic devices is likely to hamper the application of varnish on all surfaces on which *mutans streptococci* exist; thus the application of varnish should be performed before banding and bracketing in order to eliminate nearly all *mutans streptococci* at the beginning of therapy. Furthermore, the commencement of the recolonization with *mutans streptococci* after antimicrobial therapy in highly colonized teeth has to be investigated. Due to the design of the study, which assessed *mutans streptococci* counts at baseline and at 8 weeks, the definite time point of recolonization was not discernible. Adapting the repetition of varnish applications to recolonization time could presumably avoid a return of *mutans streptococci* to baseline values and achieve long-term suppression.

In contrast to the results of the present study, Øgaard *et al.* (1997) achieved *mutans streptococci* suppression in patients with fixed orthodontic appliances 20 weeks after application of a low concentration varnish. In their investigation, varnishing was performed before the placement of orthodontic bands and brackets. They found significantly reduced *mutans streptococci* counts in comparison with the untreated controls. However, those authors ignored the initial *mutans streptococci* colonization of the subjects, in contrast to the present investigation. Emilson and Lindquist (1988) evaluated the coherence of the infection level of *mutans streptococci* and recolonization of teeth after CHX treatment and reported that tooth surfaces with a high level of infection are more rapidly colonized by *mutans streptococci*, even if these micro-organisms have been suppressed to undetectable levels after antimicrobial treatment. This shows that a slow recolonization pattern and higher efficacy can be achieved after antimicrobial treatment in low colonized teeth.

Because in orthodontic patients with high caries activity standard caries prevention measures based on oral hygiene, non-cariogenic dietary habits and regular supplementation of fluorides are often insufficient to prevent the development of new carious lesions, the use of antimicrobials or, alternatively, the early removal of bands and brackets is necessary. On the other hand, the present study showed, in agreement with the findings of Jenatschke *et al.* (2001), that the efficacy of the most potent CHX treatment as shown in high-risk non-orthodontic subjects (Emilson, 1994; Pienihakkinen *et al.*, 1995; Attin *et al.*, 2003) failed in highly *mutans streptococci* colonized teeth in orthodontic patients.

Conclusions

The negative influence of orthodontic bands and brackets on the effectiveness of antimicrobial therapy could be proven in the present study. Nevertheless, the use of antimicrobials is currently the only promising alternative to improved oral hygiene, fluoridation measures and dietary counselling for caries prevention. Consideration has to be given to the mode of application of antimicrobial varnishes to achieve a long-term suppression in at-risk orthodontic patients. Developing more effective therapy modes and identifying existing sources of error in application should be investigated in further studies.

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Original Article

Antimicrobial Effectiveness of a Highly Concentrated Chlorhexidine Varnish Treatment in Teenagers with Fixed Orthodontic Appliances

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ABSTRACT

Objective: To evaluate the recolonization pattern of Mutans streptococci (*ms*) on densely colonized teeth with fixed orthodontic appliances after treatment with a highly concentrated (36%) chlorhexidine varnish.

Materials and Methods: Healthy subjects (n = 19) with fixed orthodontic appliances and high bacterial *ms* counts in saliva were recruited. In order to establish a baseline registration, plaque adjacent to brackets was sampled and cultivated on Dentocult® strips. Following professional tooth cleaning, chlorhexidine varnish was applied on all teeth for 8 minutes. The degree of recolonization with *ms* was assessed 2 weeks after varnish application in plaque around the brackets. For statistical analysis, the data were subjected to a repeated measures design.

Results: After 2 weeks, *ms* counts were reduced as compared to baseline values. However, the reduction only weakly met statistical significance ($P = .049$).

Conclusions: The application of a highly concentrated chlorhexidine varnish in patients with fixed orthodontic appliances does not result in a distinct reduction of *ms* numbers 2 weeks after treatment.

KEY WORDS: Orthodontic treatment; Caries prevention; Antibacterial varnish; Chlorhexidine; *Streptococcus mutans*

INTRODUCTION

Caries-preventive measures—good oral hygiene, establishment of noncariogenic dietary habits, and regular fluoride supplementation—often are not sufficient to prevent the occurrence of new carious lesions in orthodontic patients with high caries activity. Beyond a certain caries activity level, neither an increase in the frequency of tooth-brushing nor an increase in the dosage of administered fluoride is suitable to effectively stop the demineralization process in high-risk individuals.[1,2] Patients undergoing orthodontic therapy are subjected to oral ecologic changes that lead to increased numbers of *Streptococcus mutans* in saliva and plaque.[3,4]

It has also been shown that orthodontic treatment with fixed appliances results in enamel demineralization and an increased number of carious lesions, predominantly in sites adjacent to brackets.[5] Therefore, preventive efforts in these risk group have concentrated on direct suppression of the cariogenic microflora by chemotherapeutics as an adjunct to improved oral hygiene.

Chlorhexidine is the most potent documented antimicrobial agent against Mutans streptococci (*ms*) and dental caries. Different modes of administration are recommended for caries prevention.[6–8] It has been suggested that chlorhexidine application in the form of a varnish results in longer-lasting suppression of *ms* concentrations by chlorhexidine compared with other forms of application.[9–11] High and low concentrations have been reported to reduce the number of *ms* in plaque and saliva for considerable periods of time.[12–14]

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Numerous studies have examined highly concentrated chlorhexidine varnishes as supersaturated solutions of chlorhexidine diacetate in ethanol, stabilized by the natural resin sandarac.[15–19] In these examinations, the optimal chlorhexidine varnish concentration suggested for suppression of *ms* amounted to 36% chlorhexidine, as represented by the varnish EC40® (Dentres, Nijmegen, The Netherlands).

The consensus of these studies was that *ms* were significantly suppressed for at least 4 weeks after a single chlorhexidine varnish application. This effect has been tested on teeth free of orthodontic appliances. On the other hand, studies performed in high-risk orthodontic patients with highly concentrated varnish treatment did not show any influence on the caries increment.[20] A rapid recolonization of the teeth with *ms* and a return to baseline values was regarded as a possible reason for the failure of chlorhexidine treatment in orthodontic patients.

Orthodontic patients usually are seen at appointments every 4–6 weeks. Therefore, it is desirable that suppression of *ms* caused by antimicrobial treatment last for at least this period of time. Hence it is of interest to evaluate whether suppression of *ms* within a suitable, and for orthodontic patients common, recall interval is possible. Moreover, as yet it is not known whether highly concentrated chlorhexidine varnishes are effective in suppressing recolonization of *ms* when applied in patients with fixed orthodontic appliances.

Therefore, the objective of the present study was to investigate the time period in which *ms* in patients with densely colonized teeth and fixed orthodontic appliances return to baseline values after a single treatment with the 36% chlorhexidine varnish EC 40®.

MATERIALS AND METHODS

Participants

The participants and their guardians gave informed consent for taking part in the study. Thirty-two volunteers with fixed orthodontic appliances treated in a private practice were screened, and 19 of them (median age, 14 years) were selected. The appliances had been inserted at least 2 months prior to the start of the study. All study participants fulfilled the inclusion criteria of high levels of *ms* in saliva as demonstrated by at least a score of 2 identified with the chair-side Strip-mutans® method according to Jensen and Bratthall.[21] None of the subjects had detectable frank caries lesions or defective restorations. Moreover, clinical examination and radiographs did not reveal any lesions on interproximal tooth surfaces.

Study Design

Professional tooth cleaning was performed and oral hygiene instructions were given to the participants prior to the study. Before baseline examination, the subjects refrained from all oral hygiene measures for 24 h. At baseline, the *ms* levels in plaque and saliva were recorded.

The plaque *ms* scores were determined with the site-specific modified Strip-mutans® technique (Orion Diagnostica, Espoo, Finland) as originally described by Wallman and Krasse[22] and modified by Twetman.[31] The number of colony-forming units (CFU) with characteristic morphology was screened and scored 0–3. The evaluation was blinded. Scores were as follows:

- 0 indicates no CFU (*ms* below detection level).
- 1 indicates 1–10 CFU, corresponding to approximately $< 10^4$–10^5 CFU
- 2 indicates 10–100 CFU, corresponding to approximately 10^5–10^6 CFU
- 3 indicates $>$100 CFU, corresponding to $>10^6$ CFU.

Selected teeth for plaque sampling were isolated with cotton rolls and dried. A small sterile brush was carefully brushed on the sites around the brackets of eight teeth (teeth 11, 14, 22, 25, 31, 34, 42, and 45) in each of the 19 patients. This means that a total of 152 sites were evaluated.

All brackets had been placed by the same orthodontist with etching gel, bonding material, and light-curing composite (Transbond XT®, 3M Unitec®, Neuss, Germany). In case of an extracted premolar, the adjacent premolar was evaluated. Sampled plaque was immediately spread on the roughened side of the plastic strip from the Strip-mutans® kit (Orion Diagnostica, Espoo, Finland). The strips were allowed to dry for 5 minutes at room temperature and were then incubated for 48 hours in a liquid medium. The composition of the medium was similar to the composition of mitis salivarius agar, with a sucrose concentration increased to 30%. Addition of a bacitracin disc from the kit results in a final concentration of 0.36 U of bacitracin per ml of medium.[23] Additionally, a saliva Strip-mutans® test was performed for each participant and evaluated. After 48 hours cultivation in the liquid medium, the scores of *ms* in plaque were recorded with the aid of a stereomicroscope with 10–25$\times$ magnification.[24]

The 19 subjects fulfilling the inclusion criteria were treated with EC40® at the next recall 1 week later. EC40® is a highly concentrated chlorhexidine varnish as supersaturated solution of chlorhexidine diacetate in ethanol, stabilized by the natural resin sandarac.[17,25–28] The varnish is available in glass ampoules containing approximately 1.5 mL varnish. The ampoules fit into the normal syringe used for anesthesia. For application of varnish, a wide needle with an inner diameter of 0.8 mm. was used

Prior to each varnish application, the orthodontic

arch wire was removed and the teeth were professionally cleaned with a rubber cup and pumice paste. The interdental areas were cleaned with unwaxed dental floss. Each quadrant was isolated with cotton rolls and dried with compressed air. EC40® was applied to all teeth with a brush and delivered into the interproximal areas with unwaxed dental floss. Following the manufacturer's advice, the varnish was left in place for 8 minutes and then removed with a brush. Two weeks after varnish treatment, *ms* levels in plaque were recorded on the buccal sites as described above.

Statistical Methods

All microbiological measurements were recorded on a grading scale; the observations are so-called ordered categorical data, and thus standard statistical methods such as the *t*-test or analysis of variance cannot be applied. For the analysis of such data, ranking methods have been developed.[29] Therefore, the original observations are replaced with their ranks. Because only the four grades 0, 1, 2, and 3 are possible values, many observations will have the same values, which are called ties in the statistical literature. When ranking tied observations are given, midranks are assigned. Because ranking methods are used for the analysis of the data, it is reasonable to use the relative treatment effects to summarize the outcome of the trial in tables and graphs.

The statistical design underlying the observations in our trial is a repeated measures design, ie, the same patients are repeatedly observed at several time points. This means that statistically significant differences are assumed when time profiles are not parallel to the x-axis. For a detailed description of these methods, see Brunner et al.[30]

RESULTS

The statistical analysis was done as described above using relative treatment effects. For better illustration of the original data, the distributions of *ms* score changes during the 2-week interval are given in a cross-table (Table 1). The table demonstrates that a deterioration to higher scores occurred in 36 cases, whereas in 64 sites an improvement to lower scores took place. Scores for the other 52 sites remained unchanged. However, *ms* scores were below detection level (score 0) in only 34 sites at the end of the 2-week period.

In Figure 1, the results of site-specific plaque samples on teeth with brackets at baseline (T0) and 2 weeks (T1) after varnish treatment are demonstrated. After 2 weeks, *ms* counts were reduced as compared to baseline values. The reduction did meet statistical significance, but only weakly ($P = 0.049$). In the design of the study, we intended to record *ms* levels in plaque every 2 weeks until *ms* values returned to baseline values. Because in nearly every subject recolonization with *ms* was already complete after 2 weeks of varnish application, recording of *ms* values was not continued.

Nevertheless, we could observe intra-individual differences. The degree of reinfection varied considerably between different patients (Table 2). However, only 2 patients showed a distinct suppression of *ms* counts.

TABLE 1. Cross-tabulated Number of *ms* Values Indicating Toothwise Changes From Baseline to Two Weeks[a]

	ms Score	2 wk 0	1	2	3	Total
Baseline	0	**4**	6	5	0	15
	1	4	**6**	7	1	18
	2	11	8	**17**	17	53
	3	15	6	20	**25**	66
	Total	34	26	49	43	152

[a] Numbers set in **bold** are *ms* scores that remained unchanged during the two experimental weeks. Entries above bold numbers demonstrate numbers of sites with deterioration in *ms* scores; entries below bold numbers illustrate numbers of sites with improved *ms* scores. *ms* indicates Mutans streptococci.

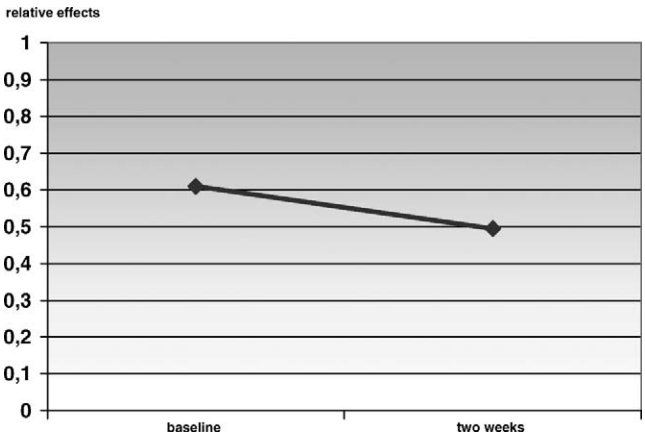

Figure 1. Relative effects of *ms* counts in plaque at baseline and 2 weeks after treatment with EC40®.

DISCUSSION

The *ms* counts in the present study were evaluated with a commercial available *S. mutans* test, namely the chair-side Strip-mutans-Dentocult® test.[31] The reliability of this method has been proven by numerous studies. There is a significant correlation between conventional analysis with MSB agar[32] and the Strip-mutans® test.[33,34] Intra-individual differences with the Strip-mutans® method were investigated by El-Nadeef

TABLE 2. Exemplary Plaque *ms* Scores (0–3) in a 13-Year-Old Girl and a 14-Year-Old Boy at Baseline and 2 Weeks After Treatment Given for the Respective Teeth at Which Plaque Samples Were Taken[a]

	13-Year-Old Girl		14-Year-Old Boy	
Tooth	Baseline	2 wk	Baseline	2 wk
14	3	1	3	3
11	3	1	2	3
22	3	2	3	3
25	2	1	3	3
34	3	2	2	3
31	2	1	1	2
42	1	1	1	2
45	1	0	3	2

[a] *ms* indicates Mutans streptococci.

and Bratthall.[35] They observed that tests that were repeated in one subject usually showed no differences. Tests varied in more than one category in very rare cases. Therefore, with good handling, the Strip-mutans® method is a very reliable method.

If fluoridation measures and dietary counseling are not considered, the use of antimicrobials is currently the only promising alternative to improved oral hygiene. However, it must be noted that studies performed in high-risk orthodontic patients did not find significant differences in caries increment after repeated application of high- or low-concentration chlorhexidine varnishes.[36,37] In contrast to these findings, other investigations with orthodontic patients have documented a reducing effect of chlorhexidine and/or fluorides on caries increment and *S. mutans*.[38–42] However, in contrast to the present investigation, in these studies patients taking part in the trials were not preselected with regard to caries risk, caries activity or levels of *ms* in plaque and saliva, respectively,

In a former study it was proved that the efficacy of a highly concentrated varnish is reduced by bands and brackets.[43] In this split-mouth–designed study, recolonization on teeth with orthodontic appliances occurred significantly faster than on teeth without appliances.

It was assumed that the duration of *ms* suppression depends partly on the extent to which any retention niches are coated with varnish. In a previous study by Jenatschke et al,[44] *ms* counts were assessed only at baseline and 8 weeks, so the definite time point of the recolonization was not discernible. In that study, the varnish was applied on the day of bracket placement and was repeated at 8-week intervals, while the fixed appliances were in place. As mentioned above, in this investigation the caries increment was not reduced despite the use of chlorhexidine varnish treatment. It can be assumed that the recolonization must have taken place during this time interval (ie, 8 weeks) so that recolonization with *ms* and caries development could not be avoided.

The recolonization with *ms* after antimicrobial therapy in highly colonized teeth was investigated in the present study. We could show that *ms* counts had nearly returned to baseline values after 2 weeks. Because only a weak significant difference between baseline and 2-week values was observed, the reduction of *ms* is assumed to be not clinically relevant. This suggestion is corroborated by the fact that most of the samples were not below detection level 2 weeks after varnish treatment.

Additionally, it could be assumed that a complete recolonization would have taken place within a short period after completion of the 2-week interval chosen in the present study. This finding may act as an explanation of why no effect on caries increment was achieved in former studies. On the other hand, an application modus applying the varnish even more often, eg, once a week, is not practicable. A more effective application mode must be found to hamper the fast recolonization of *ms* in orthodontic patients.

Emilson and Lindquist[45] evaluated the coherence of the infection level of *ms* and recolonization of teeth after chlorhexidine treatment and observed that tooth surfaces with a high level of infection are more rapidly colonized by *ms.* This was true even if these microorganisms had previously been suppressed to undetectable levels after antimicrobial treatment. This observation makes clear that a slow recolonization pattern and higher efficacy more presumably can be achieved in teeth with low levels of colonization.

Unfortunately, patients with a fast recolonization pattern are more likely to develop carious lesions after application of fixed orthodontic appliances. Therefore, an effective way of suppressing *ms* should be found for these patients. An effective way could be to apply the varnish immediately before bracketing and banding of the teeth. It may be hypothesized that this results in a slower recolonization of *ms.* Afterwards, the exact recolonization time after varnishing has to be determined to set an application interval and to suppress *ms* during the entire therapy with fixed appliances. Another focus should be set on the cleaning of the tongue, because the tongue may act as an infection source, possibly favoring the recolonization pattern on the teeth. Of course, it can also be assumed that in the present study no suppression at all could be achieved. This is speculative, because no *ms* counts were assessed right after varnish treatment. The authors had expected suppression for at least 4 weeks, and because of this assumption, the *ms* counts were investigated after a 2-week interval.

Therefore, development of more effective therapy modes and identification of reasons for lack of effec-

tiveness need to be investigated in further studies. Furthermore, interdisciplinary work between the cariologist and orthodontist should be intensified to develop caries prevention strategies for orthodontic patients, because these patients are at a high caries risk.[46]

CONCLUSIONS

- A single 36% chlorhexidine varnish application is not effective in suppression of *ms* counts to a clinically relevant degree in patients with fixed orthodontic appliances and highly colonized *ms* salivary counts.

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ORIGINAL ARTICLE

A new method for chlorhexidine (CHX) determination: CHX release after application of differently concentrated CHX-containing preparations on artificial fissures

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Abstract Aims of the study were (1) to establish a method for quantification of chlorhexidine (CHX) in small volumes and (2) to determine CHX release from differently concentrated CHX-containing preparations, varnishes, and a CHX gel applied on artificial fissures. CHX determination was conducted in a microplate reader using polystyrene wells. The reduced intensity of fluorescence of the microplates was used for CHX quantification. For verification of the technique, intra- and inter-assay coefficients of variation were calculated for graded series of CHX concentrations, and the lower limit of quantification (LLOQ) was determined. Additionally, artificial fissures were prepared in 50 bovine enamel samples, divided into five groups (A–E, n=10) and stored in distilled water (7 days); *A*: CHX-varnish EC40; B: CHX-varnish Cervitec; C: CHX-gel Chlorhexamed; D: negative control, no CHX application; and E: CXH-diacetate standard (E1, n=5) or CHX-digluconate (E2, n=5) in the solution. The specimens were brushed daily, and CHX in the solution was measured. The method showed intra- and inter-assay coefficients of variation of <10 and <20%, respectively; LLOQ was 0.91–1.22 nmol/well. The cumulative CHX release (mean±SD) during the 7 days was: EC40 (217.2±41.8 nmol), CHX-gel (31.3±8.5 nmol), Cervitec (18.6±1.7 nmol). Groups A–C revealed a significantly higher CHX release than group D and a continuous CHX-release with the highest increase from day 0 to 7 for EC40 and the lowest for Chlorhexamed. The new method is a reliable tool to quantify CHX in small volumes. Both tested varnishes demonstrate prolonged and higher CHX release from artificial fissures than the CHX-gel tested.

Keywords Varnish · Chlorhexidine · EC40 · Cervitec · Chlorhexamed gel · CHX determination

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Introduction

Chlorhexidine (CHX) is considered as one of the most popular agents to reduce the risk of developing new caries lesions [21, 26, 32]. One reason for this estimation is the broad antiseptic property of CHX against a wide variety of gram-negative and gram-positive organisms. In high concentrations, CHX is bactericidal via destruction of the cell membrane [16]. The antimicrobial activity of CHX is due

to the positively charged parts of the CHX molecule, which react with the phosphate groups of lipopolysaccharides in the bacterial cell wall. At lower concentrations, CHX has bacteriostatic properties. For use in the oral cavity, CHX is available and effective in different delivery systems, such as sprays, mouthwashes, CHX-containing glass ionomer cement gels, chips, and varnishes [1, 4, 5, 12, 19, 25, 27, 33]. Varnishes are usually applied in the dental office and act as a kind of slow-releasing device, thus resulting in a prolonged intraoral CHX availability as compared to mouthwashes and gels.

Chlorhexidine varnishes might be used for prevention of fissure caries, although the results of studies in high-risk caries patients are inconclusive [38, 39]. Especially in the time period shortly after eruption of the teeth, it is often difficult to apply adhesively attached fissure sealants due to insufficient moisture control. In this phase, CHX varnishes might help to fight bacterial inoculation of the fissure systems and to postpone application of fissure sealants to a time point when moisture control could be guaranteed.

However, application of CHX varnish onto fissures resulted in contradictory results in terms of fissure caries reduction in studies running over periods from 9 months to 3 years [39]. On the one hand, some recent split-mouth controlled studies and randomized clinical trials of various research groups [3, 7, 8, 10, 23] proved the CHX varnish Cervitec (1% CHX and 1% thymol) being effective to significantly reduce the caries incidence in molar fissures in groups of children and adolescents. In contrast, in a randomized controlled study using the 40% CHX varnish EC40, no statistically significant benefit was recorded in the group treated with the varnish as compared to the placebo group [15].

CHX has the ability to adsorb onto tooth surfaces and oral mucosa, with a slow release later on. When applied onto fissures, it is additionally possible that remnants of the applied varnish will retain even after mechanical impact, such as mastication and toothbrushing. These varnish remnants will allow for prolonged and elevated CHX levels in the fissure systems. Previous studies using Cervitec could show that this varnish is able to release CHX for as long as 3 months with the majority of release during the first 4 weeks of storage [20]. In this study, the varnish was applied on microscope glass slides (2×7.5×2.5 cm) resulting in a measurable amount of CHX release from the varnish into the 50-ml sample solution. This experimental setup allowed for determination of the CHX release with a common ultraviolet (UV) spectrophotometer. Using UV absorption for CHX determination needs both expensive quartz cuvettes and a sample volume of at least 3 ml. Additionally, application of varnishes on glass plates does not simulate intraoral conditions with mechanical impact such as toothbrushing. When applied onto the fissure system of a tooth, which is regularly brushed, only small quantities of CHX are expected to be released into the solution used to store the respective specimens. Thus, to increase the concentration in the solution, the specimens should be stored in small sample volumes. However, using the above-mentioned UV-absorption method as done in various studies [20, 28, 31], it is difficult to measure reliably such low concentrations in small sample volumes. More sensitive methods, such as high-performance liquid chromatography or ion mobility spectrometry have also been described for CHX determination [9, 11, 37, 40]. However, these approaches do need extensive sample preparation and expensive devices and does not allow remeasuring in the same sample solution, which is needed for monitoring the release over a certain period of time. Additionally, colorimetric methods using different markers for CHX, such as eosin, bromthymolblue, methylene orange and bromchresol green, have been described for CHX determination [2, 13, 18, 30, 42]. Disadvantages of these methods are either the low sensitivity or the fact that the CHX has to be extracted in the sample solution using chloroform, not allowing monitoring in the same sample solution over a period of time.

It would be useful for clinical practice to have information about the characteristics of CHX release from varnishes applied onto tooth surfaces and fissure systems.

Thus, the aims of the study were:

1) To develop and to verify an unexpensive, sensitive method for determination of low CHX concentrations in small sample volumes
2) To determine the period of time of chlorhexidine release from CHX varnishes applied on artificial fissures subjected to toothbrushing

Materials and methods

Principle of the method to quantify CHX in small sample volumes

The following principle for quantification of CHX in small volumes was developed in the laboratory of the authors. The determination of CHX was conducted using polystyrene flat-bottom microplate wells (Sarstedt, Nümbrecht, Germany) in a microplate reader (SpectraMax M2, Molecular Devices, Ismaning/Munich, Germany). When excited with 280 nm from the top, the bottom of the polysterene plate shows fluorescence, resulting in light emittance with a wavelength of 360–380 nm. Chlorhexidine is able to absorb light with the wavelength of 280 nm during the passage but not the emitted light, resulting in a decreased intensity of

the fluorescence of the polystyrene (measured as RFU, relative fluorescence intensity; Fig. 1). When calculating the relation between the RFU of a blank well and a well containing a CHX solution excited at 280 nm, the best results are achieved at emitted fluorescence of 370 nm. This finding indicates the high sensitivity to determine CHX when measuring intensity of emitted fluorescence at 370 nm (Fig. 1).

In a first step, the unfilled polystyrene wells of the microplates were excited with 280 nm, and the emitted fluorescence was recorded at 370 nm to determine the blank value. Then, 370 µl each (1 cm height in a well) of a standard dilution series with 0, 7, 14, 28, 56, 111, and 223 µmol/l CHX was applied into the wells to create a standard calibration curve. The final content of CHX in the respective wells corresponded to 0, 2.6, 5.2, 10.3, 20.6, 41.2, and 82.4 nmol CHX per well. The standard calibration curve was fitted with polygonal interpolation. Relative fluorescence was expressed as percentage of the blank value and was recorded. By means of the standard calibration curve, the absolute quantity of CHX (nmol) per well is determined. Since the CHX regimes later used in the study contained different CHX formulations, standard calibration curves were recorded for both CHX-diacetate and CHX-digluconate. No differences were observed for these two compounds with respect to the calibration curves recorded (Fig. 2). Owing to that finding, CHX-diacetate was used in the study later on for measuring the CHX release from the CHX-treated specimens.

Validation of the method

Precision, reproducibility and lower limit of quantification (LLOQ) were checked according to the guidance for bio-analytical method validation recently described by Shah et al. [34, 35]. The measurements were performed at room temperature of 25°C. The experiments were run with ten repeats in series using graded dilution series of CHX-diacatate from 0.7 to 78.4 nmol/well. Either 100, 200 or 370 µl were pipetted into each well.

Intra-assay coefficient of variation of CHX recovery was calculated for assessing precision of the test since it considers both distribution of the data and slope of the calibration curve. Threshold for acceptable precision was set at a coefficient of variation of <10%. For example, an intra-assay coefficient of variation of 10% would mean that a CHX concentration of 100 µmol/l in a respective solution would yield a reading in a range of 90–110 µmol/l.

Reproducibility was checked by calculating the inter-assay coefficient of variation. Therefore, for each solution,

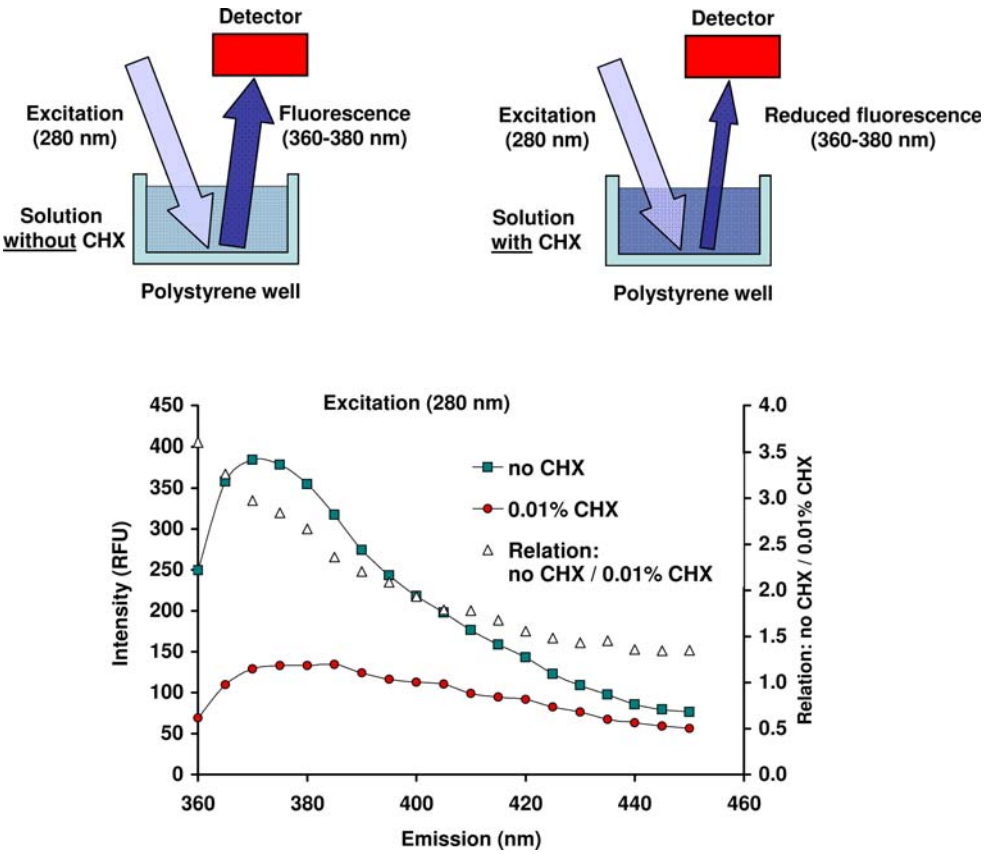

Fig. 1 *Above*, schematic drawing of the principle of the CHX-determination (*right*) measuring the reduced fluorescence of the polysterene plates due to absorbance of the excitation light by the CHX in the solution (*left*). *Below*, intensity (relative fluorescence units, RFU) of emission wavelengths (nm) determining a solution without (no CHX) and with 0.01% CHX in the polysterene plates excited with 280 nm. Additionally, the relations between the fluorescence intensity measured for the solution without and with 0.01% CHX are presented

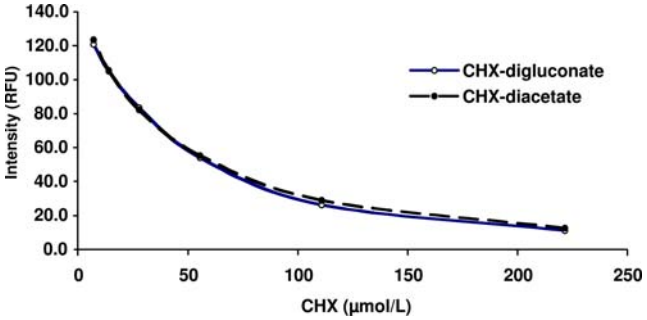

Fig. 2 Mean intensity (relative fluorescence units, RFU) of differently concentrated solutions of CHX-digluconate and CHX-diacatate

ten calibration curves were constructed, and recovery of CHX and inter-assay coefficient of variation was calculated. Threshold for acceptable reproducibility was set at an inter-assay coefficient of variation of <20%. For example, an inter-assay coefficient of variation of <20% would mean that using different calibration curves a CHX concentration of 100 µmol/l in a respective solution would yield a result of 80–120 µmol/l.

Lower limits of quantification (nmol/well) for the respective solutions were calculated as CHX concentration at the particular point on the calibration curve presenting the following: (mean value of blank fluorescence) minus (5× standard deviation). As a threshold, the lowest standard on the calibration curve (2.6 nmol/well) should be higher than the LLOQ [34, 35].

Preparation of enamel samples

Fifty bovine central lower incisors were used in the study. After extraction, the teeth had been stored in 0.1% thymol until use. Cylindrical samples (5 mm in diameter) were prepared from the labial surface with a diamond-coated trephine drill (Geb. Brasseler/Komet, Lemgo, Germany). The cylinders were then embedded in acrylic resin (Technovit 4071®, Kulzer, Wehrheim, Germany). Subsequently, the specimens were ground flat with water-cooled carborundum discs (500–4,000 grit; Water Proof Silicon Carbide Paper, Struers, Erkrath, Germany). Grinding and polishing resulted in approximately 100 µm of the enamel being removed. The amount of abraded enamel was controlled with a micrometer (Digimatic; Mitutoyo-Meßgeräte, Leonberg, Germany).

Using a diamond-coated disc (Komet), grooves (0.5 mm in depth and 0.2 mm in width) were prepared on the polished surface under constant water-cooling. The grooves were arranged in the form of four crosses. A special device designed for this purpose was utilized to guarantee standardized preparation of the grooves. After cleaning the artificial fissures with water and air-drying, the samples were divided into five groups (A–E) of ten samples each:

– Group A (CHX varnish EC40)

The varnish EC40 (explore, NL-6501, Nijmegen, Netherlands) contains 40% CHX-diacetate in a sandarac resin base, dissolved in water-free alcohol and is packed in glass carpules. The varnish was applied onto the enamel surface of the samples in a thin layer of about 1 mm using a syringe with a blunt needle.

After setting of the varnish for 10 min, gross material excess was removed with a probe as instructed by the manufacturer. Subsequently, the surfaces of the enamel samples were brushed with 40 strokes in an automatic brushing machine [41]. The toothbrush Oral-B classic soft (Procter & Gamble, Schwalbach am Taunus, Germany) with a load of 200 g was used in a toothpaste slurry consisting of the dentifrice elmex (Gaba, Münchenstein, Switzerland) dissolved in distilled water in a ratio of 1 g:3 ml. The samples were then stored in 700 µl distilled water in closed polyethylene containers for 7 days and brushed every day.

– Group B (CHX varnish Cervitec)

Cervitec (Ivoclar Vivadent, Schaan, Liechtenstein) contains 1% CHX-diacetate and 1% thymol dissolved in ethanol, ethyl acetate and a polymer (polyvinyl butyral), and is packed in glass vials (1.5 ml). The user's instruction recommends the first toothbrushing not until 24 h after application of the varnish. The varnish was applied in two layers onto the enamel samples with a microbrush.

The samples were also transferred to a vial containing 700 µl of distilled water. The first brushing was conducted after a period of 24 h storage. Thereafter, the samples were brushed every day as described for group A.

– Group C (CHX gel Chlorhexamed)

The Chlorhexamed gel (GlaxoSmithKline, Bühl, Germany) contains 1% CHX-digluconate 1%. The gel was applied with a microbrush onto the enamel surfaces in a layer of about 1 mm thickness. First brushing of the samples was conducted after the application of the gel, before transferral to the storage media. Thereafter, the samples were brushed every day as described for group A.

– Group D (negative controls)

Neither gel nor varnish was applied on the enamel samples. These samples were brushed and were stored in 700 µl distilled water also.

– Group E

No application of CHX onto the enamel surface was done, like in group D. However, these samples were not brushed and were subdivided into two subgroups (E1 and E2) of five specimens each with respect to the storage media. In group E1, samples were stored in 700 µl of a solution containing 55.4 µmol/l chlorhexidine-diacetate (i.e., 38.8 nmol CHX-diacetate). In group E2, the storage media contained 55.7 µmol/l chlorhexidine-digluconate in the 700 µl solution (i.e., 39.0 nmol CHX-digluconate). Groups E1 and E2 were included in the study for checking adsorption of the different CHX compounds to the enamel

surface. The values recorded for these samples were later used to calculate the net release from the different CHX preparations.

Determination of CHX in the storage media

As mentioned above, each sample was stored in 700 µl distilled water for a period of 7 days. On each day, 370 µl were pipetted from the solution 3 h after brushing and used for determination of CHX in the microplate reader as described above. After determination, the 370 µl liquid sample was retransferred to the vials containing the enamel samples. Thus, at the end of the experiment, CHX measurement in the solution represented the cumulative CHX release over the 7-day period. The values recorded for each day were corrected by the value obtained in group E (adsorption to enamel surface), so that the net release of the CHX regimes was used in the statistical analysis and presentation of the data in the result section.

The determination gave the absolute amount of CHX (nmol) in the 370 µl samples. With this value, the absolute amount of CHX (nmol)] released from the CHX-treated enamel samples into the 700 µl storage solution was calculated.

Statistical analysis

Comparisons between the different groups regarding CHX release directly after application of the regimes were performed with one-way analysis of variance (ANOVA). The increase of CHX release over time (starting after day 0 to day 7) was conducted with a weighted one-way ANOVA and post hoc-tests with adjustments according to Bonferroni–Dunn. The level of significance was set at $P<0.05$.

Results

Validation of the method for CHX-determination

The recovery rate of CHX measured in different volumes (100, 200, and 370 µl) with 0.7–78.4 nmol CHX per well ranged from 99.1 to 100.4%. In Figs. 3 and 4, the intra- and inter-assay coefficients of variation of the CHX determination with different concentrations and different volumes are given. The intra-assay coefficients of variation amounted to <10% for most of the solutions tested except for levels below 2.8 nmol/well in 100 or 200 µl and below 3.5 nmol/well in 370 µl solution. All inter-assay coefficients of variation were below the threshold level of 20%. Lower limits of quantification for the different volumes applied to the wells of the microtiter plate were calculated as follows: 1.22 nmol/well (100 µl), 0.91 nmol/well (200 µl) and 1.01 nmol/well (370 µl). Thus, the requirement was fulfilled that the LLOQ should be lower than the lowest standard on the calibration curve (2.6 nmol/well).

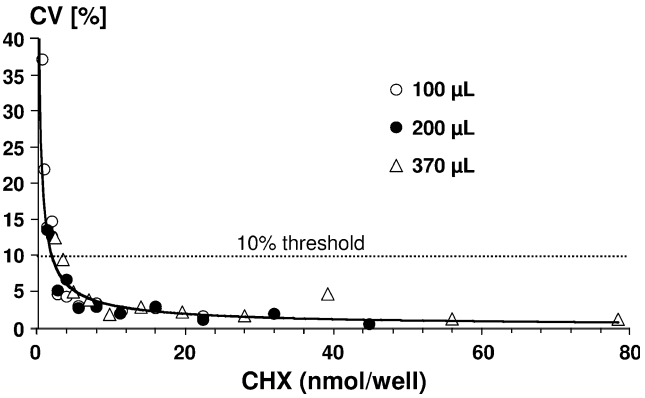

Fig. 3 Intra-assay coefficients of variation (CV%) for different CHX amounts (nmol/well) measured in volumes of 100, 200, and 370 µl in the wells of microtiter plates. The 10% threshold area is marked with a *dotted line*

CHX-determinations in test and control samples

The control group did not show any measurable amounts of CHX in the solutions at any time during the experiment. The samples of group E showed a loss of both CHX-diacatate (E1) and CHX-digluconate (E2) from the solutions during the 7-day period. From the 38.8 nmol of CHX-diacetate and 39.0 nmol CHX-digluconate in the solutions, only 22.9±4.9 nmol (E1) and 22.4±8.5 nmol (E2) could be recovered on day 7. This finding indicated that about 41–42% of the CHX from these solutions were adsorbed on the enamel surfaces during the 7-day period.

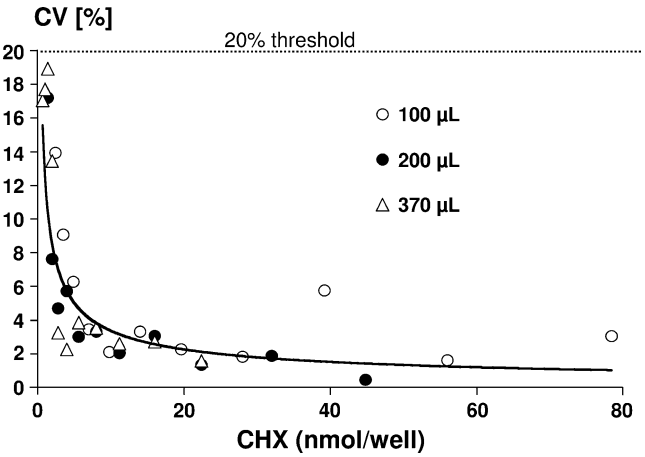

Fig. 4 Inter-assay coefficients of variation (CV%) for different CHX amounts (nmol/well) determined in volumes of 100, 200, and 370 µl in the wells of microtiter plates. The 20% threshold area is marked with a *dotted line*

In Fig. 5, the cumulative CHX net release from the different regimes are given. Due to the different CHX concentrations of the regimes, significantly different amounts of CHX were released especially directly after application. The release directly after application at day 0 was therefore statistically significantly highest for EC40 as compared to the two other treatments ($P<0.0001$). The varnish Cervitec released the significantly lowest amount of CHX ($P<0.0001$). For better comparison of the CHX release over time, the cumulative CHX release after day 0 to 7 was additionally calculated. This means that the values of day 1 to 7 were added. All test groups showed a continuous release of CHX after day 0 to 7 with the statistically highest increase for EC40 and the lowest for Chlorhexamed. The increases from day 0 to 7 and the P values of the increases were as follows: EC40 (174.3±26 nmol; $P<0.0001$), Cervitec (17.9±1.6 nmol; $P<0.0001$) and Chlorhexamed (7.4±5.1 nmol; $P=0.0025$).

Discussion

The new method for CHX determination fulfilled all requirements for bioanalytical measurements as described by [35, 34]. All parameters checked, such as intra- and inter-assay coefficients of variance as well as the recovery rate and the lower limit of quantification, proved the method being suitable for detection of low CHX-concentrations in small sample volumes. The LLOQ calculated was in a range of about 1.0 nmol/well. Thus, the LLOQ fell below the CHX release per day measured in a pilot study for samples treated with the CHX gel Chlorhexamed. To increase the amount of CHX in the storage solutions, we, therefore, had decided to measure the CHX release in the same solution during the 7-day experimental period. This means that the CHX-treated samples were not transferred to a fresh solution for measuring daily release, as done in studies determining release of other substances from dental materials, such as fluoride release from glass ionomer cements [6, 14, 24, 29]. The same procedure of measuring CHX in the identical solution during storage was done by Huizinga et al. [20], when determining CHX release from Cervitec during 16 weeks. Due to this necessity, no daily release of CHX could be determined, and a cumulative release over time was determined. This renders it nearly impossible to estimate the CHX release from the enamel samples with respect to antimicrobial potential in the in vivo situation. Additionally, for the situation in the oral cavity, it has to be considered that CHX, which is released from a carrier, is diluted or bound to various surfaces and might interact with salivary proteins and salivary bacteria. Nevertheless, in the present study, the cumulative release for all forms of CHX application was above the minimal inhibitory concentration (MIC) of CHX, which is given as 0.19–2.0 μg/ml [17]. Taking the molar weight of 506 for CHX, the MIC would equal 0.26–2.76 nmols in our storage solution volume of 0.7 ml. This value was exceeded by both the varnishes and the gel at least directly after application. Even the approximate daily release from the two varnishes was above this calculated MIC. Nevertheless, extrapolation of the obtained in vitro data to the clinical situation with respect to possible bacterial inhibition should be done with caution.

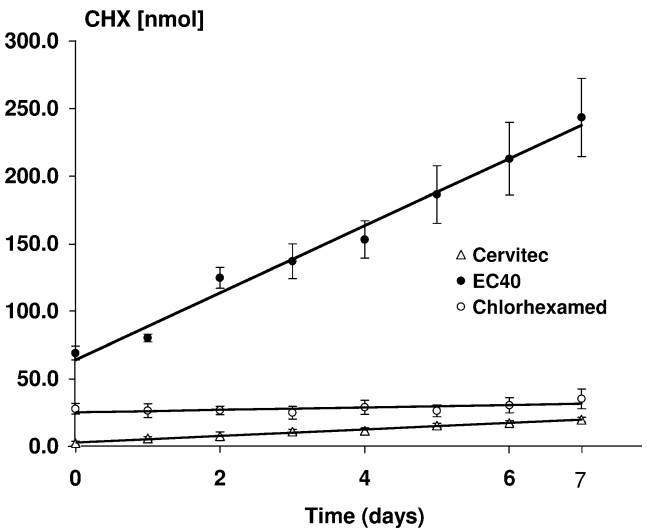

Fig. 5 Cumulative mean (and SD) of net CHX (nmol) recorded in the storage solutions containing the enamel samples either treated with Cervitec, EC40, or Chlorhexamed. The CHX determined in the storage solution is given for each day of the 7-day-period of storage in the same solution. A regression line is drawn for each CHX application

The results of the study showed clearly that the two CHX varnishes tested have the potential to release measurable amounts of CHX after application to an artificial fissure system which is brushed regularly. Furthermore, after application of the Chlorhexamed gel, a constant although lower release as compared to the varnishes was recorded. The higher CHX release from the varnish EC40 as compared to Cervitec might be attributed to the higher concentration of the EC40. This becomes obvious when referring to the CHX release immediately after application. However, also in the period after day 1, the daily release was higher from EC40 represented by the steeper slope of the regression line of EC40 as compared to Cervitec (Fig. 5). Nevertheless, in contrast to the CHX gel, also Cervitec showed retention in the fissure system under the simulated toothbrushing procedure resulting in a prolonged and steady CHX release after application of the varnish onto the fissures. The better retention of EC40 in contrast to Cervitec might be attributed to the higher stickiness of EC40 and the fact that EC40 hardens to a solid consistency difficult to remove from the fissures by toothbrushing.

The study is the first study measuring CHX release after application onto a fissure system. The extensions of the fissures were prepared as previously done by Smits and Arends [36] in an in vitro study. Use of artificial fissures was necessary in our study to standardize the determination of CHX release. However, it should be noted that natural fissures might have various shapes, thus allowing different retention of a varnish applied and different access of toothbrush bristles. This means that retention of the tested CHX regimes to natural fissures might, in some part, be different as compared to the present in vitro situation.

The present study was limited to a period of 7 days. For a better understanding of the CHX action in the oral cavity, a longer observation period would be desirable. In a future study, it should be clarified how long the two varnishes tested are able to show a measurable release of CHX. However, antimicrobial therapies utilizing CHX for reducing caries risk are mostly limited to a certain period, ranging from a single application of a highly concentrated product to multiple applications of lower concentrated gels during this treatment period [7, 10, 15, 22, 43]. These restricted periods seem to be sufficient to reduce the critical mass of carious-inducing bacteria for a certain period of time, as shown in numerous previous studies. Thus, it could be assumed that the CHX release as observed in the present study for 7 days could contribute to reducing the carious risk in fissures. Nevertheless, repeated applications after a certain period of time of at least 6 months seem to be advisable [26].

In conclusion, the study proved the newly introduced method as a reliable tool to detect and quantify minimal CHX contents in small volumes. Additionally, under the chosen conditions, both tested varnishes demonstrated prolonged and higher CHX release from artificial fissures in comparison to the CHX gel tested.

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